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14. ABSTRACT

The *long-term goal* of our research program is to identify the key molecular events that lead to development and progression of breast cancer, in order that improved therapies to treat this disease can be developed. The *objective* of studies outlined in this proposal is to determine if sustained inhibition of annexin II prevents breast cancer cell growth and angiogenesis. During the performance period of this grant, we have developed a novel nanotechnology mediated vector (nanoparticles) for delivering a novel protein annexin II. We show that nanoparticle mediated delivery of human annexin II inhibitory RNA results in reduced cell proliferation and cell migration. Future studies should be aimed at testing the ability of these sustained release nanoparticles on reducing breast cancer tumors in an animal model system.

15. SUBJECT TERMS
Annexin II, Nanoparticles, angiogenesis, tumors

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INTRODUCTION

The *long-term goal* of our research program is to identify the key molecular events that lead to development and progression of breast cancer, in order that improved therapies to treat this disease can be developed. The *objective* of studies outlined in this proposal is to determine if sustained inhibition of annexin II prevents breast cancer growth and angiogenesis. Our *central hypothesis* is that the nanoparticle-mediated sustained expression of an inhibitory RNA (RNAi) for annexin II results in inhibition of breast cancer cell proliferation and angiogenesis. A non-viral gene delivery technology that provides sustained release of an important gene in breast carcinogenesis would provide potential new opportunities for the development of therapeutic strategies to treat breast cancer. We propose to determine the effect of nanoparticle-mediated annexin II delivery on growth and angiogenesis of breast tumors in a mouse model system. The working hypothesis for this aim is that sustained delivery of annexin II-RNAi to breast tumors in mice leads to reduction in tumor number and volume, and prolongs animal survival.

BODY

Plasmid DNA Preparation

The plasmid DNA was expanded in E. Coli (DH5α) and selected by using a appropriate antibiotic. The plasmid DNA was extracted by QIAGEN Plasmid Giga Kit. The purity of the DNA was confirmed by agarose electrophoresis. The concentration of the DNA was determined by UV spectrometry at 260 nm.

For expression of full-length annexin II, we cloned the full-length annexin II cDNA into the vector pcDNA3.1 as shown in Figure 1. When expression of annexin II had to be tracked with a fluorescent tag, we developed the vector pEGFP-C1-AnxII in which annexin II is produced as a fusion protein with the green fluorescent protein at the N-terminus (Figure 2).

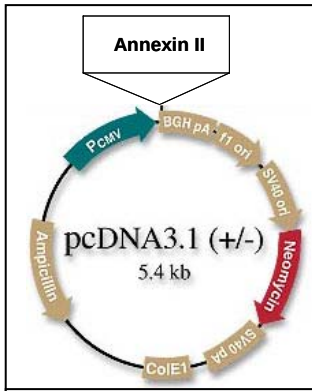


Figure 1: Plasmid map of pcDNA-WT Anx II

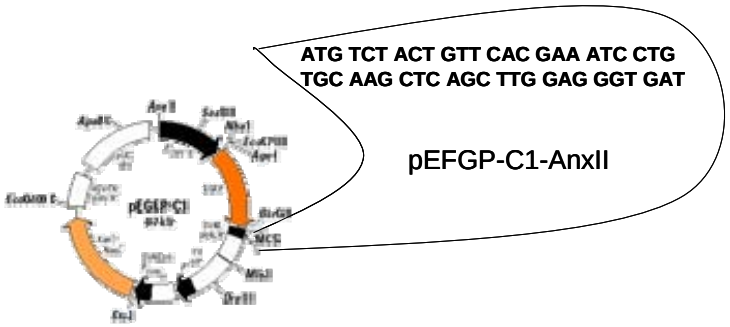


Figure 2: Plasmid map of pEGFP-C1-AnxII

To determine the effect of annexin II downregulation, we developed the pDrive-shAnxII vector (Figure 3). First we synthesized a number of double stranded small inhibitory oligoucleotides

corresponding to human annexin II sequence and tested their ability to inhibit annexin II expression. From these initial studies, we developed one double stranded oligonucleotide that efficiently inhibited annexin II expression. This oligonulcleotide was inserted into the pDrive vector that will produce small hairpin oligonucleotides upon transfection of cells.

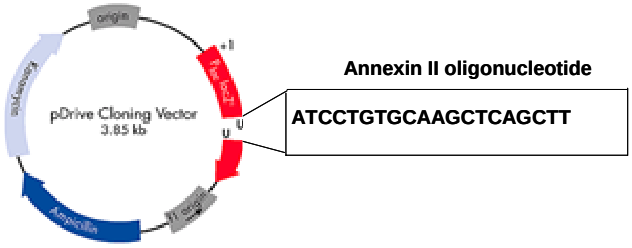


Figure 3: Plasmid Map of pDrive-sh-Anx II

Characterization of the plasmid DNA-loaded nanoparticles.

The emulsion-solvent evaporation yielded both blank and plasmid-loaded blend particles with sizes around 300 nm (Figure 4). Generally, the encapsulation of plasmid DNA caused slight changes in the size of the nanoparticles (Figure 4). It was shown that all plasmids had good capacity of incorporating PLGA polymer, with the entrapment efficiency ranging from 48.89% to 74.63%. pEGFP-C1, pEGFP-C1-WT-Anx II, pCDNA-WT-Anx II and pDrive-sh-Anx II have the sizes of 4.7 Kbp, 5.7 Kbp, 6.4 Kbp, and 3.8 Kbp, respectively. We observed that the entrapment efficiency is positively correlated with the size of the plasmid (Figure 4). This observation suggests that the smaller the size of the DNA plasmid, the easier can it be incorporated into the PLGA nanoparticles. The nanoparticles containing these plasmids showed good polydispersity index around 0.13 (Figure 4), which suggested that the particles were homogenous, and along with the negative zeta potentials, also suggested that these stable and homologous nanoparticle suspensions, when used in vivo, may show stable effects in transfection of cancer cells.

In vitro release of the encapsulated plasmids

In general, macromolecules are released from biodegradable matrices by a mechanism involving polymer degradation, followed by matrix erosion, and diffusion of the macromolecules through the aqueous channels generated during the erosion process (Csaba et al., 2005). Typically, the release of an encapsulated macromolecule from PLGA nanoparticles follows a triphasic release pattern where the initial phase corresponds to the detachment of the surface-localized drug molecules. The second phase, characterized by limited release, is prolonged until significant PLGA degradation and matrix erosion take place. Once this critical point is reached, the encapsulated drug releases suddenly, very often in a pulsatile manner (Blanco and Alonso, 1998). In this study, we suspended the DNA nanoparticles in phosphate buffered saline (PBS), and the suspensions were allowed to be mixed in 37ºC orbital shaker. At defined time intervals, the suspensions were collected for measuring the concentration of released DNA using the picogreen reagent which is a specific fluorescent cyanine dye for double stranded DNA. The DNA release profiles are shown in Figure 5. The linear results of the DNA releasing assay showed that the PLGA nanoparticles released the encapsulated plasmids in a sustained manner. As we noted, the rates of releasing are different among different plasmids, and the higher the molecular weight of the plasmid, the lower the releasing rate of the corresponding

nanoparticles. This might be due to the channel sizes formed during the erosion of the nanoparticle, which at first, were only easy for small sized plasmids to get through.

Particle type	particle size(nm)	P.I.	Zeta potential (mV)	DNA entrapment efficiency(%)
pEGFP-C1	332.12 ± 1.21	0.15 ± 0.03	-15.26 ± 2.15	72.42
pEGFP-C1-WT-AnxII	323.82 ± 2.02	0.11 ± 0.03	-12.75 ± 4.06	53.92
pCDNA-WT-AnxII	331.52 ± 4.13	0.12 ± 0.05	- 45.10 ± 4.13	48.89
pDrive-Sh-AnxII	306.16 ± 6.07	0.14 ± 0.04	-25.98 ± 0.76	74.63
Control(without DNA)	262.88 ± 3.28	0.16±0.02	-17.89 ± 6.63	0

Figure 4. Mean particle size, Polydispersity Index, Zeta-potential and Entrapment efficiency of the PLGA : PVA nanoparticles loaded with our without DNA

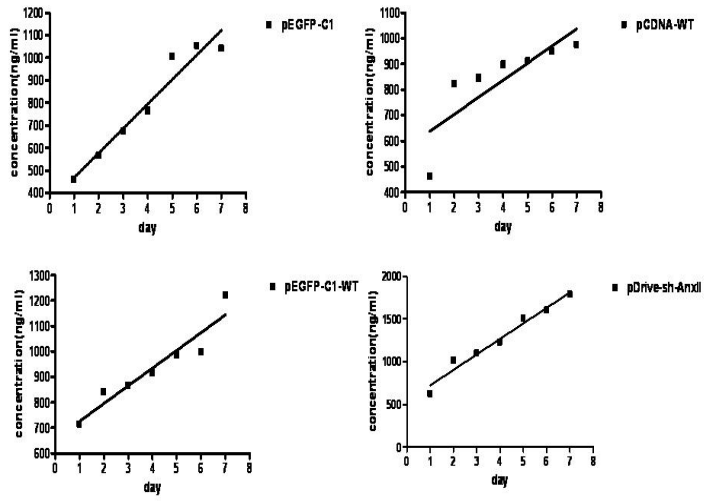


Figure 5. Release profiles of plasmids from PLGA nanoparticles. Nanoparticles containing different plasmids (pEGFP-C1, pEGP-C1-WT-Anx II, pCDNA-WT-Anx II and pDrive-sh-Anx II) were subject to in vitro releasing assay as described in methods. The DNA concentrations in the supernatants were measured using PicoGreen reagents which is specific for double strand DNA molecules. The data were analyzed using GraphPad Prism 3.0.

Structural integrity of the encapsulated plasmid DNA

One of the major challenges of plasmid DNA encapsulation into polymeric vehicles is the preservation of its structural integrity and, thus of its biological activity (Csaba et al., 2005). The quantification of the supernatants of the release samples was performed using the PicoGreen reagent which is a specific fluorescent cyanine dye for double stranded DNA. However, apart from the conservation of the double stranded structure, it is also necessary to maintain the biologically active form of the plasmid molecule. Therefore, we examined the samples from the releasing assay by agarose gel electrophoresis and we have compared them with the intact plasmid (Figure 6). Biologically active plasmid DNA is present in two forms, supercoiled form, which migrates faster than its linear counterpart in agarose, and open circular form which migrates much slower than its linear counterpart. In Figure 6, the plasmids released from the nanoparticles were in the open circular form (Figure 6) compared with the intact plasmids (Figure 6). On the other hand, no conversion to the linear form could be detected in the release samples. This observation was very important for the linear form is the biologically less active one and more susceptible to further degradation and fragmentation than the circular forms.

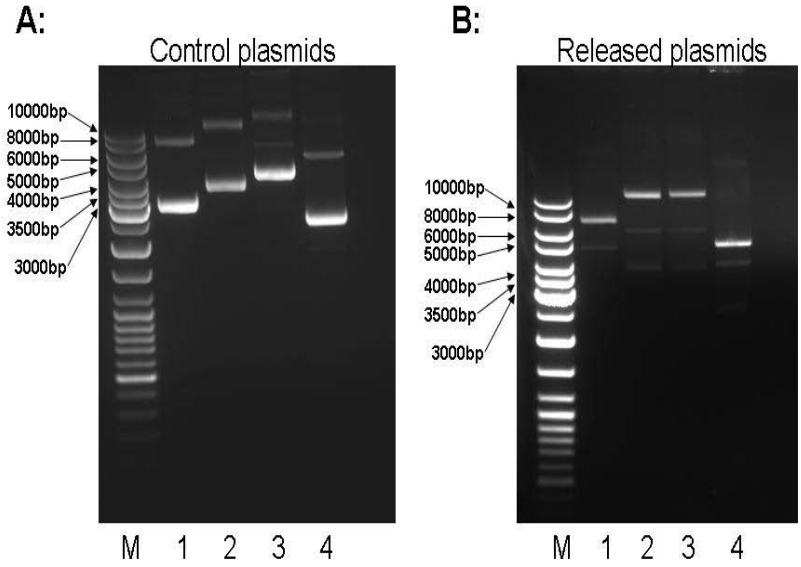


Figure 6. Agarose gel electrophoresis of plasmid DNA released from PLGA nanoparticles. (A). Intact control plasmids. Lane M: maker; lane 1, 2, 3, 4: pEGFP-C1, pEGFP-C1-WT-Anx II, pCDNA-WT-Anx II and pDrive-Sh-Anx II, respectively. (B). Released plasmids. Lane M: maker; lane 1, 2, 3, 4: pEGFP-C1, pEGFP-C1-WT-Anx II, pCDNA-WT-Anx II and pDrive-Sh-Anx II, respectively.

Alternate Formulation and Characterization of annexin II-RNAi loaded Nanoparticles

We investigated the formulation parameters for the formation of poly-lactide-co-glycolide (PLGA) nanoparticles. Nanoparticles are formulated from a FDA-approved, biodegradable and biocompatible polymer, poly (DL-lactide-co-glycolide) (PLGA), that undergoes slow hydrolysis within the cell to release the gene at a sustained rate (Panyam and Labhasetwar, 2003). Investigations were performed such that we would have the flexibility to generate nanoparticles of any desired size range for encapsulation of plasmid DNA or proteins. Current methodologies employ the use of a primary emulsion followed by sonication and stabilization of the nanoparticles using the amphiphillic agent polyvinyl alcohol (PVA) (Sahoo et al., 2002). Addition of sonic energy generates a very fine emulsion but there is the problem of shearing or linearization of plasmid DNA and as such the input of sonic energy has to be kept at a minimum. Figure 7, panel B illustrates nanoparticles formed using this existing strategy. The size range of these particles is centered around 400-500 nm, with 2 minutes of sonic energy input. Reviews of current literature suggest that optimum transfection of plasmid DNA loaded PLGA nanoparticles

occurs when the population size is below 300 nm. In an effort to control the ultimate size of the nanoparticle and reduce the input of sonic energy required we initiated the use of a non-solvent system. Figure 7, panel A shows the results of nanoparticle formulation using DMSO as the PLGA solvent and methanol as the non-solvent. Under these formulation conditions we are able to form stable nanoparticles centered around 100 nm, with sonication time of no more than 45 seconds. It is also evident that the size distribution of nanoparticles formed through this method is much less polydisperse than particles formed using sonication alone, Figure 4, panel B. The change of solvent to chloroform and non-solvent to ethanol leads to the formation of particles centered around 250 nm Figure 7 panel C. Approximately 90 percent of the nanoparticle population is below the 300 nm cellular uptake cut-off. Sonic energy input is less than 45 seconds. We have selected the use of nanoparticles formulated by this method as optimum in terms of loading capability and transfection potential. Additionally use of ethanol as the non-solvent assists in minimizing cytotoxcicity of the nanoparticles. This method of formulation could very easily be applied to the capture of proteins in nanoparticle drug delivery systems.

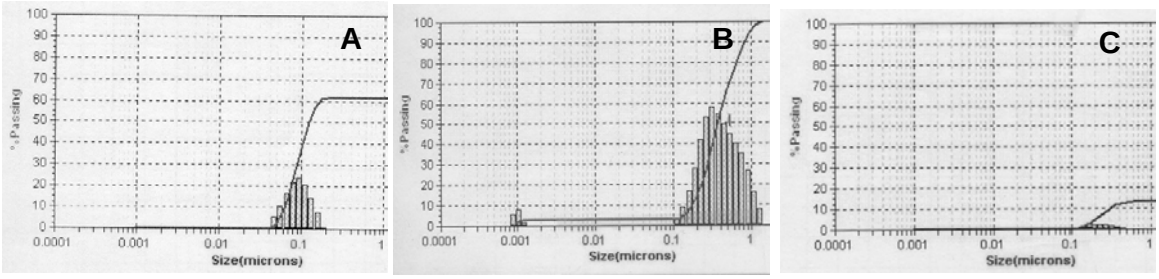


Figure 7: Formulation parameters and their effect on size of plasmid DNA loaded nanoparticles. Through the optimal choice of solvent/non-solvent systems we can control the size of nanoparticles produced. Panel A: Solvent: DMSO, Non-solvent: Methanol; size range 50-100 nm. Panel B: Solvent: Chloroform, Non-solvent: Chloroform; size range 100-1000 nm. Panel C: Solvent: Chloroform, Non-solvent: Ethanol; size range 100-400 nm.

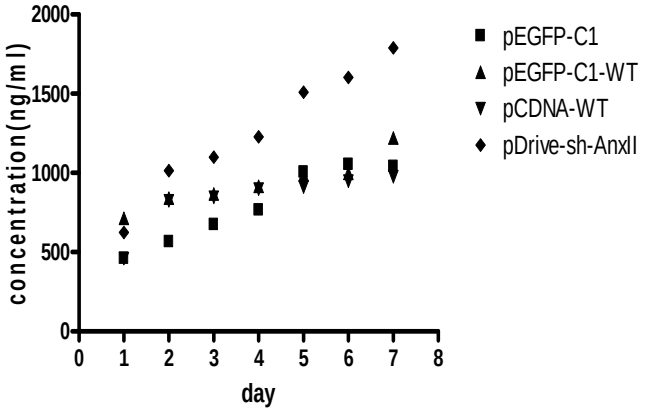
Next, we determined the DNA incorporation into the nanoparticles that were formed. Data in Table 1 shows two different batches of nanoparticles and percent incorporation of DNA.

Table 1: Incorporation efficiency of DNA into PLGA Nanoparticles.

DNA Nanoparticle	Amount of DNA Loaded	Incorporated DNA	Entrapment Efficiency
pcDNA 3.1– Anx II	1 mg	670 µg	67.00%
pDrive-sh Anx II #1	1 mg	820 µg	82.00%
pEGFP	1 mg	665 µg	66.50%
pEGFP-WT	1 mg	662.5 µg	66.25%
pCDNA-WT	1 mg	416.5 µg	41.65%
pDrive-sh Anx II #2	1 mg	564.5 µg	54.65%
Control	0 mg	0 µg	100%

Having established formulation parameters for the production of our nanoparticles we then characterized the release of encapsulated plasmid DNA to verify both sustained release of DNA and plasmid integrity. Figure 8 illustrates that our nanoparticles show a slow linear release of the encapsulated plasmid DNA over a 7 day time period. Our entrapment efficiency is greater than 90%.

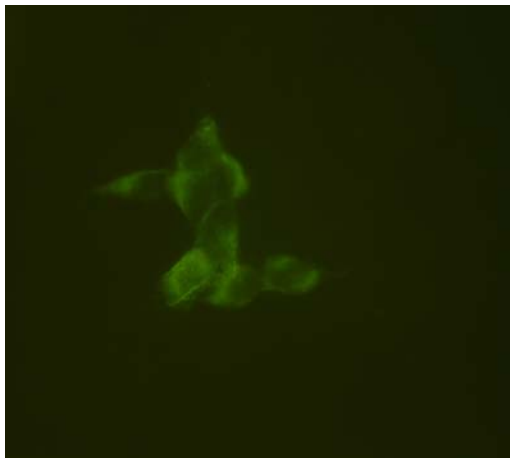
Figure 8: Cumulative release of plasmid DNA from PLGA nanoparticles in ng/ml over a 7 day period. A slow linear release is observed independent of the loaded plasmid DNA.



Uptake of nanoparticles and effect on cell viability

Having established control over the size and polydispersity of the nanoparticles and verified the integrity and release rates of encapsulated plasmid DNA, we first sought to determine the transfection capabilities of our nanoparticles. To determine the intracellular localization of our nanoparticles we reacted FITC with glutamine and co-stabilized the nanoparticles with PVA and FITC-glutamine. Breast cancer cells were then transfected for 1 hour and visualization of the particles was done using fluorescence microscopy. Figure 9 shows the results of these experiments.

Figure 9: FITC labeled nanoparticles are capable of cellular transfection. Nanoparticles co-stabilized with glutamine labeled FITC and PVA demonstrate rapid cellular uptake and transfection levels greater than 90%. Endosomal localization of the nanoparticles can be observed.

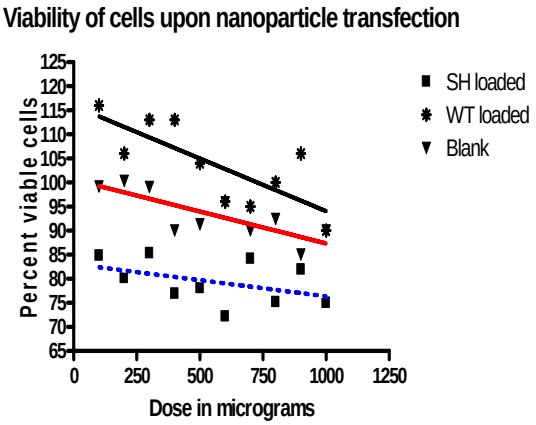


For determination of transfection efficiency, 5 cellular fields were examined and the average number of fluorescent cells was counted. We have found that greater than 90 percent of the cells demonstrated uptake of the fluorescent nanoparticles. It is also possible to see intracellular localization to endosomes within 1 hour of exposure to nanoparticles. Our current focus with regard to transfection of cells with nanoparticles is to determine the transcriptional activation of

delivered plasmid DNA. This will be accomplished through loading of nanoparticles with a GFP containing plasmid.

Having shown the ability to successfully transfect cell lines with our nanoparticles we then sought to determine the viability of a given cell type upon transfection with plasmid DNA loaded nanoparticles. Delivery of wild type annexin II, blank and pDrive Sh nanoparticles showed differential effects on cell viability. It can be seen from the figure that blank nanoparticles display a minimal reduction in the percent of viable cells. For nanoparticles that contain the wild type plasmid DNA a dose dependent reduction in cell viability is observed. These results imply that the reduction in cell viability is not due to the effect of the nanoparticles themselves. In this set of experiments delivery of wild type annexin II leads to overexpression of Annexin II protein. Delivery of pDrive Sh nanoparticles leads to the production of a small inhibitory RNA against Annexin II protein synthesis in the nucleus. Figure 10 illustrates the results of these transfection experiments.

Figure 10: Cell viability after transfection with various nanoparticles. Transfection of cells with nanoparticles loaded with plasmid DNA for the production of an inhibitory RNA molecule show decreased viability (blue line). Transfection with blank nanoparticles show sustained viability (red line). Transfection with nanoparticles loaded with wt annexin II show increased viability (black line).



Effect of annexin II nanoparticles on cell proliferation

We have established that there is a very minimal impact of the polymeric nanoparticle itself on cellular viability. Therefore, it is our conclusion that there is successful transcription of delivered plasmid DNA into the cellular environments and it is the delivery of the plasmid DNA that has the impact upon cellular viability and proliferation. With this information at hand we investigated the impact of plasmid DNA delivery to cells on the rates of cellular proliferation. We delivered wild type plasmid DNA from nanoparticles at two concentrations. Figure 11 shows the results of nanoparticle mediated delivery of wild type annexin II plasmid DNA.

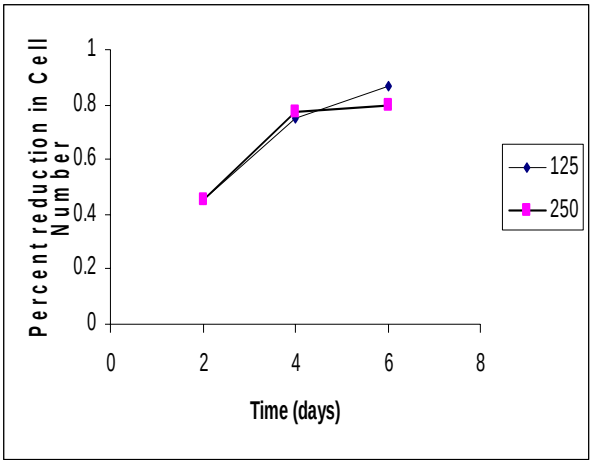


Figure 11: Reduction in cell proliferation upon exposure to wt plasmid DNA loaded nanoparticles. WT annexin II plasmid DNA loaded nanoparticles were used at concentrations of 125 μg and 250 μg of nanoparticles per mL. An increase in the reduction of cells is observed over 6 days.

Here we observe a reduction up to 80 percent in cell number over 6 days. The reduction is seen to be linear in nature and concentration independent.

Effect of annexin II nanoparticles on cell migration

It is the goal of our investigations to apply the *in vitro* experiments to an *in vivo* model for a reduction in breast tumor volume. However, due to the limited time and budget, we were unable to initiate these experiments. As a final indicator of the successful application of plasmid DNA loaded nanoparticles for this endeavor we performed cellular migration experiments. In these experiments annexin II expression was down-regulated with pDrive Sh nanoparticles for 4 days. Cells were then trypsinized and mixed in a 0.2% agar solution and plated on fibronectin coated plates. Following 24 hours of incubation at 37^0C cell were visualized. Figure 12 shows the results of this set of experiments. From the figure it is evident that there is a limited effect of blank nanoparticles upon cellular migration, panel C. It is also clearly visible that nanoparticle mediated transfection of pDrive Sh has a significant impact on cellular migration, panel B.

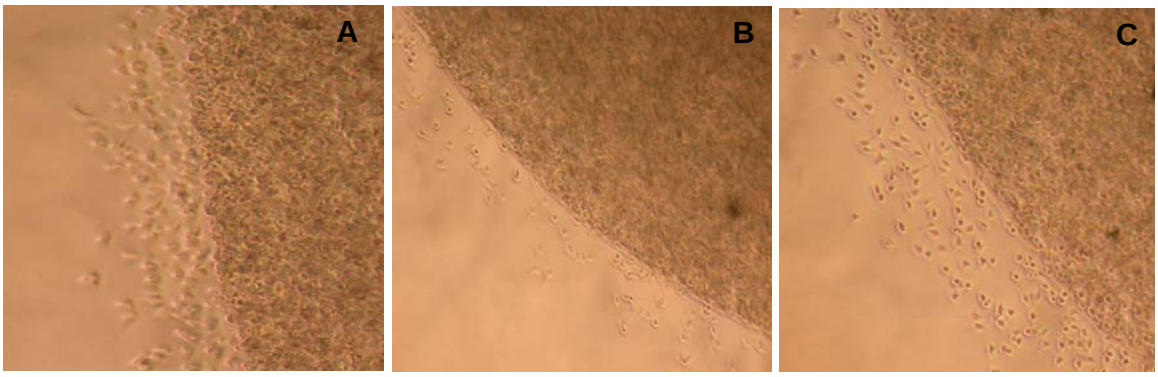


Figure 12: Migration of cells upon administration of pDrive sh plasmid DNA loaded nanoparticles and blank nanoparticles. Transfection of cells was performed for 4 days and visualized 24 hours after plating of the migration assay. Control cells are seen in panel A. There is a tremendous reduction in cellular migration with plasmid loaded nanoparticles (panel B). There is no effect upon migration when treated with blank nanoparticles (panel C).

KEY RESEARCH ACCOMPLISHMENTS

We have successfully formulated plasmid DNA loaded sustained release nanoparticles. We have established formulation parameters that allow us to control the size and polydispersity of the nanoparticles. Our formulation parameters can be very easily adapted for the capture of proteins and cell specific targeting. We have demonstrated that there is negligible cytotoxicity associated with blank nanoparticles. Blank nanoparticles do not have an impact upon cell viability, proliferation or cellular migration. We find that nanoparticles loaded with plasmid DNA do have an impact on prostate cancer cell lines. Annexin II overexpression results in a decrease in cellular viability and cellular proliferation. In data not shown, we have seen a decrease in cellular migration upon both wild type annexin II nanoparticle mediated delivery and a nuclear export mutant of annexin II nanoparticle mediated delivery. We have seen inhibition of Annexin II production upon nanoparticle mediated transfection with pDrive Sh. We have shown a decrease in cell viability, proliferation and cellular migration.

REPORTABLE OUTCOMES

Abstracts:

1. Regulation Of Breast Cancer Cell Growth And Migration By Annexin Ii Nanoparticles, Jie Liu, Jaspreet Vasir, Vinod Labhasetwar And Jamboor Vishwanatha. Abstract presented at the Era of Hope meeting, Philadephia,PA, June 2005.

2. Polymeric Nanoparticle Mediated Inhibition of Annexin II Leads To A Reduction Of Cellular Proliferation And Migration**,** *Arthur R. Braden, Erica Kafka, Jamboor K. Vishwanatha. Abstract presented at the UNTHSC Annual Research Appreciation Day, Fort Worth, TX, April 2006.*

CONCLUSION

We have demonstrated successful formulation of annexin II-loaded sustained release PLGA-nanoparticles. In cell culture systems, we have shown these particles to be effective in reducing cancer cell proliferation, cell viability and cell migration. In future experiments, the ability of these nanoparticles to inhibit tumor formation in an animal model system needs to be evaluated.

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